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Immunization of Macaques with Live Simian Human Immunodeficiency Virus (SHIV) Vaccines Conferred Protection Against AIDS Induced by Homologous and Heterologous SHIVs and Simian Immunodeficiency Virus

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To evaluate the vaccine potential of SHIVs attenuated by deletion of viral accessory genes, seven rhesus macaques were sequentially immunized with Δ*vpu*Δ*nef* SHIV-4 (vaccine-I) followed by Δ*vpu*SHIV$_{PPC}$ (vaccine-II). Despite the absence of virological evidence of productive infection with the vaccine strains, based on analysis of infectivity among peripheral blood mononuclear cells (PBMC) of the vaccinated animals, all seven animals developed binding as well as neutralizing antibodies against both vaccine-I and -II. The animals also developed vaccine virus-specific CTLs that recognized homologous as well as heterologous pathogenic SHIVs and SIV, and also soluble inhibitory factors that blocked the *in vitro* replication of the vaccine strains and different challenge viruses. Virus-specific cellular and humoral responses were sustained throughout a 58-week prechallenge period. To model aspects of natural transmission, the animals received a mucosal (rectal) challenge, with a mixture of three challenge viruses, SHIV$_{KU}$, SHIV$_{89.6}$P, and SIV$_{mac}$R71/17E. Two mock-vaccinated control animals inoculated with the same mixture of challenge viruses developed large numbers of infectious PBMC, high plasma viremia, and precipitous loss of CD4$^+$ T cells. The control animals did not develop any immune responses and succumbed to AIDS between 6 and 7 weeks postchallenge. All seven vaccinated animals became infected with challenge viruses as indicated by the presence of infectious cells in the PBMC and/or viral RNA in plasma. However, peak plasma viremia in vaccinates was two to nearly five logs lower than in the control animals and later plasma viral RNA became undetectable in all vaccinates. Vaccinated animals maintained normal CD4$^+$ T cell levels throughout the study. Challenge with pathogenic viruses caused massive anamnestic responses as determined by quantitation of virus-specific CD4$^+$ and CD8$^+$ T cells by intracellular IFN-γ staining, and these cells persisted for at least 74 weeks. The study is still in progress and at this time DNA of SIV has become undetectable in lymph nodes of six of the seven vaccinates, SHIV$_{89.6}$P in five of the seven, and SHIV$_{KU}$ in three of the seven animals. © 2002 Elsevier Science (USA)

INTRODUCTION

The slow progress in development of a successful AIDS vaccine may be attributed, in part, to the fact that definitive immunological correlates of protection are not completely understood (Letvin, 1998) and that HIV continues to grow in the presence of vigorous immune response by constantly evolving mutant viruses that escape preexisting immune responses (McMichael, 1998; Stipp *et al.,* 2000; Allen *et al.,* 2000a,b). However, it is now becoming clear that virus-specific cytotoxic T lymphocytes (CTLs) can play a major role in controlling productive infection. Recently, it has been shown that CD8$^+$ T cell depletion in SIV-infected rhesus macaques resulted in massive enhancement of viral replication (Schmitz *et al.,* 1999; Jin *et al.,* 1999). This has strengthened a consensus that a suitable vaccine must be capable of inducing strong CTL responses. Virus-specific neutralizing antibodies may also be valuable since passive immunization studies have shown that these antibodies prevent infection (Burton *et al.,* 2001; Parren and Burton, 2001; Mascola *et al.,* 1999, 2000; Shibata *et al.,* 1999).

Pathogenic chimeric simian human immunodeficiency viruses (SHIV) have been extensively used as challenge viruses to study the biology and efficacy of candidate vaccines in macaque model of human AIDS (Amara *et al.,* 2001; Barouch *et al.,* 2000, 2001; Villinger *et al.,* 2000; Chen *et al.,* 2001; Belyakov *et al.,* 2001; Habel *et al.,* 2000; Matano *et al.,* 2001). These viruses were typically prepared by serial passage of molecular constructs of SHIVs in macaques. The pathogenic derivative viruses caused disseminated infection characterized by high-level viremia and explosive replication in CD4$^+$ T cells resulting in nearly total loss of this lymphocyte population (Joag *et al.,* 1996, 1997a,b, 1999; Luciw *et al.,* 1995; Reimann *et al.,* 1996a,b; Igarashi *et al.,* 1999).

The live-attenuated vaccine approach is one of several strategies being pursued to develop a successful vac-

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cine against AIDS (Letvin, 1998; Kumar and Narayan, 2001; Johnson and Desrosiers, 1998). This concept of vaccine development is based on observations by several groups that live-attenuated SIV strains, unlike pathogenic SIV, replicated at a very low level and induced strong and long-lasting immune responses. Immunized animals were highly resistant to the effects of pathogenic viruses given as challenge (Daniel *et al.,* 1992; Johnson *et al.,* 1997, 1999; Connor *et al.,* 1998; Gauduin *et al.,* 1999; Shibata *et al.,* 1997). Our choice of live-attenuated vaccine was based on the observations of genetic changes in *vpu* and *nef* genes that occurred in SHIV-4 during the animal passage which yielded virulent $SHIV_{KU-1}$ (McCormick-Davis *et al.,* 1998). Assuming that changes in two auxiliary genes *vpu* and *nef* were vital for development of the virulent virus, we deleted portions of both genes from original SHIV-4 and used this virus as vaccine-I (Δ*vpu*Δ*nef* SHIV-4) (Joag *et al.,* 1998b). We also selected another nonpathogenic virus ($SHIV_{PPC}$) and deleted 60 bp from the *vpu* gene and used this also as a live-attenuated vaccine (Δ*vpu*$SHIV_{PPC}$, vaccine-II) (Joag *et al.,* 1998b). Our earlier studies with these vaccine viruses showed that vaccine-I did not productively replicate in pig-tailed animals but it induced responses that caused delay in onset of disease from infection by $SHIV_{KU-1}$, given as challenge. However, these animals eventually succumbed to AIDS-related complications within 2 years after challenge (Stipp *et al.,* 2000). Vaccine-II on the other hand, replicated productively for a transient period and induced long-lasting protection against pathogenic $SHIV_{KU}$-induced AIDS. These animals have remained healthy for more than 3.5 years following challenge (Joag *et al.,* 1998b; Silverstein *et al.,* 2000).

Since the success of the vaccine-II was thought to be linked to its prior phase of productive replication, during which it had the benefit of the presence of large amounts of viral proteins to mount strong cellular immune responses, we designed another study to ask whether prior infection in macaques with vaccine-I, followed by inoculation with vaccine-II, would result in reduction of the productive phase of replication of vaccine-II, and whether such animals would have the same beneficial effects of vaccine-II as seen in the earlier study. Seven macaques were used in the study, and following immunization, were challenged rectally with a mixture of three pathogenic viruses, comprising homologous $SHIV_{KU-2}$ and 2 heterologous pathogens, $SHIV_{89.6}P$ and $SIV_{mac}R71/17E$, a neurovirulent derivative of $SIV_{mac}251$ (Sharma *et al.,* 1992).

RESULTS

Vaccine phase

Macaques immunized with vaccine-I followed by vaccine-II did not develop productive infection in peripheral blood mononuclear cells (PBMC) or lose $CD4^+$ T cells. Seven adult rhesus macaques were immunized subcutaneously with vaccine-I (10^4 $TCID_{50}$) followed by oral inoculation of 10^4 $TCID_{50}$ of vaccine-II 4 months later. Two control macaques were mock immunized with culture medium. All nine animals were monitored for approximately 2 years for different hematological, immunological, and virological parameters. None of the animals developed infectious PBMC in the blood after inoculation with vaccine-I or vaccine-II. To ensure that vaccine-II did cause infection, seven vaccine animals were again inoculated with 10^4 $TCID_{50}$ of vaccine-II by subcutaneous route, 7 months after the first vaccine inoculation. However, none of the vaccinated animals developed infectious PBMC in the blood. Nevertheless, viral infection had been established in all seven animals because SIVgag DNA was detected in PBMC by PCR. The animals were also monitored for $CD4^+$ T cell counts at 4-week intervals, and none showed any loss. The mock-immunized animals were negative for SIVgag DNA and vaccine virus-specific antibodies, and they maintained normal $CD4^+$ T cell profiles during the prechallenge phase (results not shown).

Development of binding and neutralizing antibodies in vaccinated and control macaques. Plasma samples were examined for the presence of binding and neutralizing antibodies 4 and 13 weeks after the first vaccination (vaccine-I), at two time points (week 19 and 26) after oral inoculation of vaccine-II, and at four time points (weeks 38, 54, 70, and 93) beginning 12 weeks after the second vaccination with vaccine-II (Fig. 1). Vaccine-I induced binding antibodies in all seven vaccinated animals and was first detected at 4 weeks after inoculation. Vaccine-I Env-specific antibody titers increased after vaccine-II boost and showed peak values between weeks 26 and 38. These antibodies persisted throughout the vaccine phase. All seven vaccinated animals also developed vaccine-II Env-specific binding antibodies that became detectable soon after the first vaccine-II inoculation. Vaccine-I- and -II-specific antibodies bound with the envelope of homologous challenge virus ($SHIV_{KU-2}$). Plasma samples from five animals also recognized $SHIV_{89.6}P$ envelope, but binding was significantly lower. These vaccine-induced antibodies did not bind with $SIV_{mac}R71/17E$ envelope.

All seven animals developed vaccine-I-specific neutralizing antibodies that continued to increase after vaccine-II boosts. Vaccine-II-specific neutralizing antibodies became detectable in six of the seven animals, but only after the second inoculation with vaccine-II. However, neutralizing antibody titers against vaccine-II were lower compared to those against vaccine-I. Following administration of the second dose of vaccine-II, five of the seven animals developed cross-neutralizing antibody titers against $SHIV_{KU-2}$ and two of these had lower titers against $SHIV_{89.6}P$. However, none of the vaccinated animals developed neutralizing antibodies against

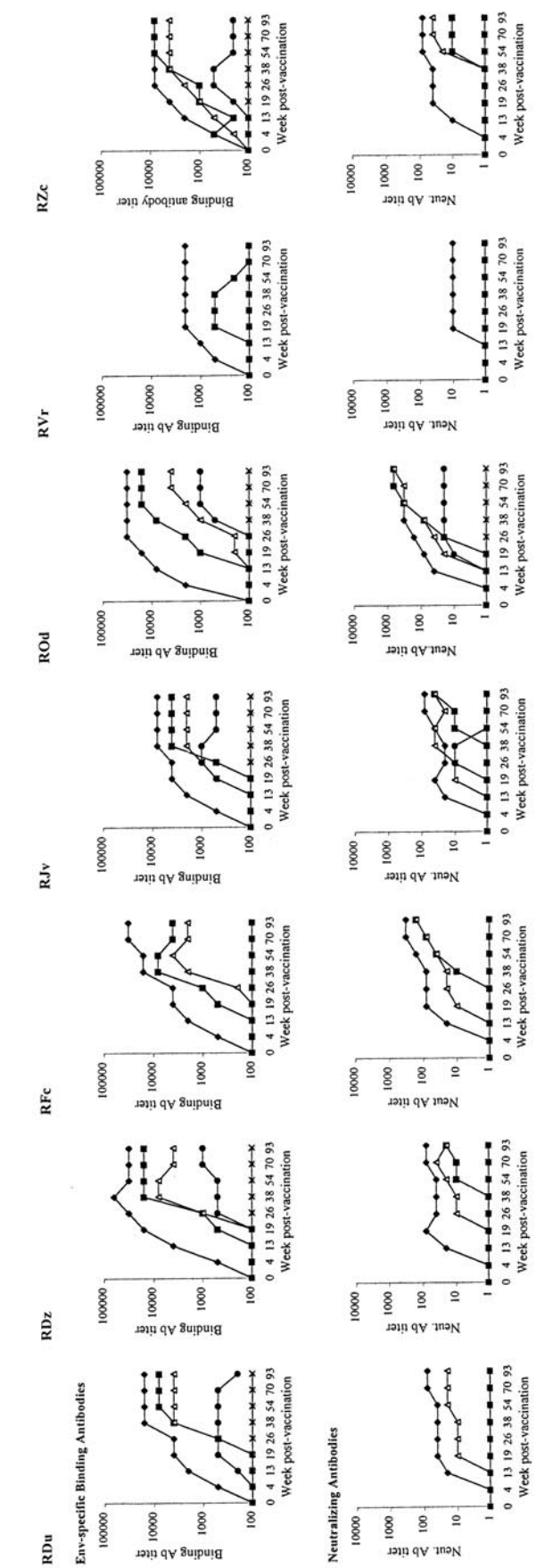

FIG. 1. Development of viral Envelope-specific binding (top), and vaccine and challenge virus-specific neutralizing antibodies (bottom) in seven rhesus macaques immunized sequentially with vaccine-I followed by vaccine-II. Sequentially collected plasma samples were tested at different dilutions in Con A ELISA using density gradient purified and Triton X-100 vaccine and challenge virus as source of binding antigen as described under Materials and Methods. Results are presented as end point titers against vaccine-I (◆), vaccine-II (□), $SHIV_{KU-2}$ (△), $SHIV_{89.6}P$ (●), and $SIV_{mac}R71/17E$ (×). Neutralizing antibody titers were determined by preincubating different dilutions of sequentially collected plasma samples in quadruplicate with different vaccine and challenge viruses, followed by culture with C8166 cells for 7 days. The wells were scored for CPE and results are presented as end point neutralizing antibody titers against vaccine-I (◆), vaccine-II (□), $SHIV_{KU-2}$ (△), $SHIV_{89.6}P$ (●), and $SIV_{mac}R71/17E$ (×).

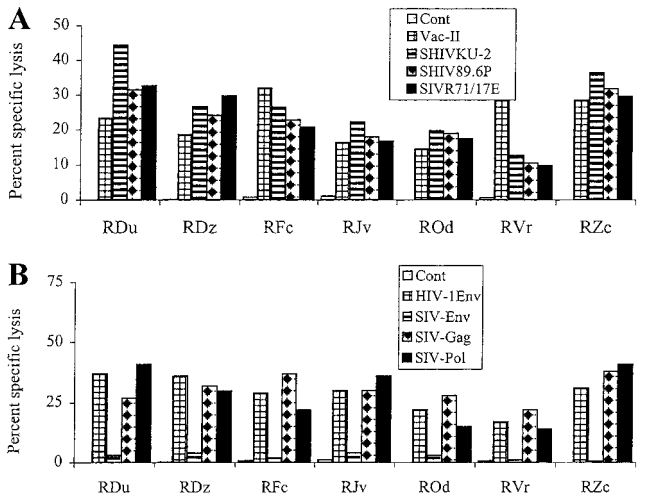

FIG. 2. CTL mediated lysis of autologous target cells infected with vaccine-II and challenge viruses (A), and recombinant vaccinia viruses expressing different viral proteins (B). The CTL effectors were prepared from the seven vaccinated animals by cocultivation of PBMC, obtained 40 weeks after second vaccine-II inoculation, with UV irradiated autologous HS-CD4$^+$ T cell clones infected with vaccine-II as described under Materials and Methods. They were tested after 2 weeks of initial stimulation against mock-infected T cells and autologous T cells infected with vaccine-II, SHIV$_{KU-2}$, SHIV$_{89.6}$P, and SIV$_{mac}$R71/17E as well as recombinant vaccinia viruses expressing HIV-1Env, SIV-Env, SIV-Gag, and SIV-Pol. The effectors were tested in a 4-h chromium release assay at E/T ratio of 80/1, 40/1, 20/1, and 10/1. Specific lyses obtained at E/T ratio of 40/1 is shown above. The mean spontaneous counts in these experiments were between 62 and 986 and the mean maximal release between 416 and 9864.

SIV$_{mac}$R71/17E. Two control animals did not develop any neutralizing antibodies (results not shown).

Development of vaccine-specific CTLs that recognized autologous CD4$^+$ T cells infected with SHIV$_{KU-2}$, SHIV$_{89.6}$P, and SIV. We tested PBMC at 40 weeks after the last immunization with vaccine-II (68 weeks after vaccine-I) for their potential to lyse autologous *Herpesvirus saimiri* (HS)-immortalized CD4$^+$ T cells infected with vaccine-II virus homologous pathogenic virus SHIV$_{KU-2}$, heterologous pathogenic virus SHIV$_{89.6}$P, and SIV$_{mac}$R71/17E. The results of these experiments are shown in Fig. 2. The effectors from all seven vaccinates recognized all four viruses and caused specific lysis of targets infected with the viruses. As expected, effectors from different macaques displayed different degrees of lysis; macaque RDu was the best, followed by RZc, RFc, RDz, RJv, ROd, and RVr, respectively. In view of the results that CTLs from different vaccinated animals recognized four different viruses that contained different envelopes, we next determined whether vaccine-induced CTLs were directed only against one protein or different proteins encoded by the virus. To further characterize the antigen specificity of the response, we used the same CTL effectors and tested them against autologous B-LCL targets infected with recombinant vaccinia viruses expressing HIV-1Env, SIV$_{mac}$239-Env, SIV-Gag, and SIV-Pol. The results of these experiments are illustrated in Fig. 2B. The PBMC effectors from various animals showed specific lysis against HIV-1Env, SIV-Gag, and SIV-Pol, but effectors from none of the seven animals caused significant lysis against SIV-Env (Fig. 2B). PBMC from the two control macaques did not have CTL activity (data not shown).

Vaccinated animals developed soluble virus inhibitory factors. The vaccinated animals developed soluble viral inhibitory factors that lasted for at least 59 weeks after the last vaccine inoculation. Resting PBMC from different macaques were stimulated with UV-irradiated autologous HS-transformed CD4$^+$ T cells infected with vaccine virus for 3 days. After 3 days of stimulation, exogenous IL-2 was added to the cultures and the cells were allowed to grow for 4 additional days. On day 7 poststimulation, the supernatant fluids were collected and tested for their ability to inhibit replication of vaccine-II, SHIV$_{KU-2}$, SHIV$_{89.6}$P, and SIV$_{mac}$R71/17E. The results from these experiments are shown in Fig. 3. PBMC supernatant from macaque RDu had the maximum inhibitory activity against all four viruses, followed by RFc, RVr, RZc, ROd, RDz, and RJv, but none of the supernatant fluids (except RDu) showed complete inhibition of any of the viruses. Supernatant fluids from mock-vaccinated macaques RKk and REs did not have inhibitory activity against any virus.

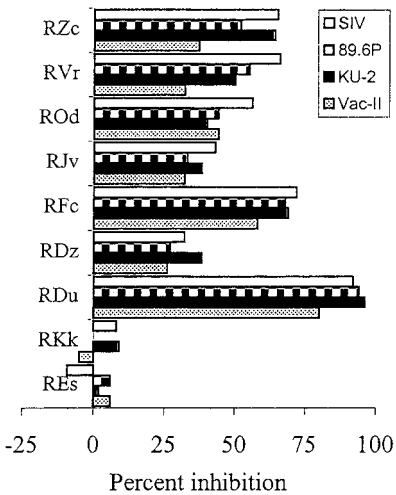

FIG. 3. Antigen-induced soluble viral inhibitory factor(s) from PBMC of seven rhesus macaques immunized sequentially with vaccine-I followed by vaccine-II and two control macaques. The soluble factors were prepared from the seven vaccinated and two control animals by cocultivation of PBMC, obtained 40 weeks after second vaccine-II inoculation, with UV irradiated autologous HS-CD4$^+$ T cell clones infected with vaccine-II as described under Materials and Methods. Culture supernatants were collected on day 7, filtered through 0.22-μM membrane, and tested for their ability to block *in vitro* replication of vaccine-II, SHIV$_{KU-2}$, SHIV$_{89.6}$P, and SIV$_{mac}$R71/17E in CEM cells. Virus replication in CEM cells was determined by measuring RT activity in 30 μl supernatants. The results presented here depict inhibition in percentage considering virus production in the absence of PBMC supernatants as 100%.

Challenge phase

Clinical outcome of challenge in control animals. The two control and seven immunized animals were challenged 65 weeks after the last vaccine dose by intrarectal inoculation of a virus cocktail containing more than 100 animal infectious doses of each of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$, respectively. A second intrarectal challenge of the same cocktail was given 24 h later. The two mock-vaccinated controls (REs and RKk) developed highly productive infections as determined by infectious cell assays (Fig. 4A) and plasma viral loads (Fig. 4B), and lost >95% $CD4^+$ T cells within 3 weeks postchallenge (Fig. 4C). On day 14, they had 2.1×10^7 and 4.9×10^7 viral RNA copies/ml of plasma, respectively. In subsequent weeks, both animals showed nearly total loss of $CD4^+$ T cells from the PBMC. However, viral RNA loads remained high. These animals progressed to clinical AIDS and lost >25% body weight within 6 weeks after challenge. Both animals developed neurological signs (hyperreactivity, movement-coordination, hand tremor, and balance). They were euthanized at weeks 6 and 7, and histopathological examination of their tissues showed meningitis, mild encephalitis, and severe depletion of $CD4^+$ T cells from lymphoid tissues. Infectious virus was isolated from PBMC, lymph node cells, and from 10% homogenates of spleen, lung, liver, and brain. PCR analyses of total cellular DNA from different organs showed the presence of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$ DNA in all organs except brain. Remarkably, PCR examination of nine regions of the brains of these two animals showed the presence of only $SIV_{mac}R71/17E$ DNA (results not shown). The reason for this exclusive infection in the CNS by SIV is not clear.

Clinical outcome of the challenge in vaccinated animals. All seven vaccinated macaques developed productive virus replication in the PBMC after challenge with $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$. These animals showed peak virus replication at week 2 to 3 postchallenge, and the number of infectious cells ranged between 10 and 10,000/10^6 PBMC (Fig. 5A). Viral RNA in plasma was detectable only in six of the seven animals (Fig. 5B) and peak viral loads ranged between 770 and 1.7×10^5 RNA copies/ml. There was a general correlation between infectious PBMC and plasma viral RNA since peak values were observed at the same time points. After ICA tests became negative, the plasma viral RNA loads fell below 10^4 copies Eq/ml. Three animals (RFc, RVr, and RZc) controlled the productive virus replication quickly and viral RNA became undetectable by week 4. ROd had a peak viral load of 5100 copies/ml and showed productive virus replication until week 6 postchallenge. The two other vaccinates, RDz and RJv, had peak viral loads of 1.7 and 1.6×10^5 copies/ml, respectively, and showed productive virus replication for at least 28 weeks after challenge. Viral RNA was not detected

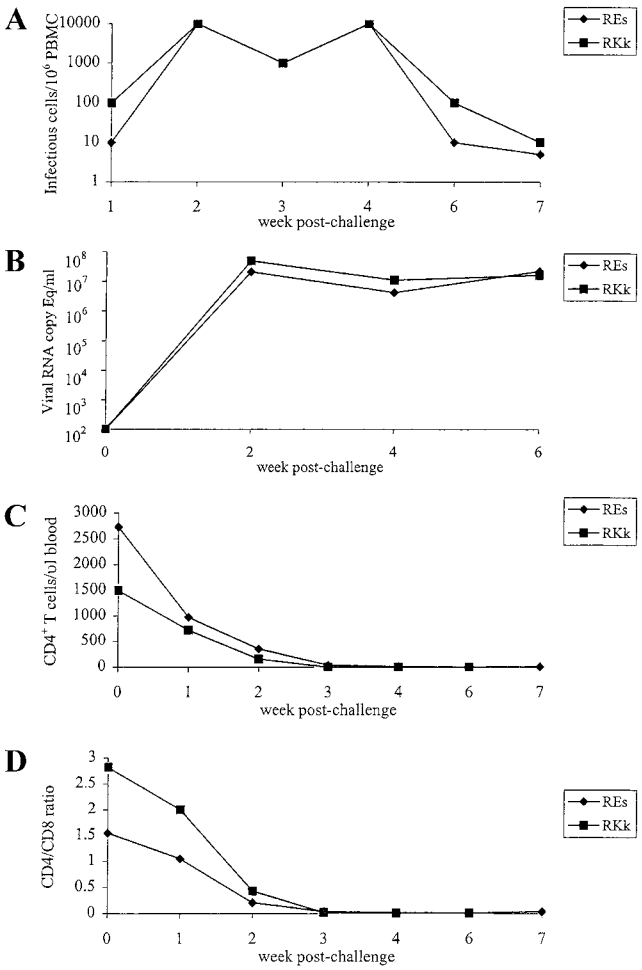

FIG. 4. Cell-associated virus burden, plasma viral RNA loads, $CD4^+$ T cell profile, and CD4/CD8 ratio in two control macaques challenged with cocktail of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$. Two macaques, RKk and REs, were mock immunized and challenged intrarectally twice, 1 day apart, with mixture of three pathogenic viruses. Cell-associated viral burdens were measured by determining frequency of infectious cells/10^6 PBMC (A), and viral RNA loads in plasma determined by real time RT-PCR (B) as described under Materials and Methods. $CD4^+$ T cell counts (C) were monitored by staining with mixture of MAbs directed against CD3, CD4, and CD8 molecules. The absolute number of $CD4^+$ T cells/μl of blood was calculated by multiplying the percentage of lymphocyte subset with absolute number of lymphocytes/μl of blood from complete blood count (CBC). CD4/CD8 ratio (D) was calculated by dividing absolute number of $CD4^+$ T cells by absolute numbers of $CD8^+$ T cells.

from any of the plasma samples taken from the seventh vaccinate, Rdu. At week 61, when the last real time RT-PCR analysis was performed, macaque RJv was the only vaccinated animal that was positive for viral RNA (440 copies/ml plasma) (Fig. 5B). None of these vaccinated animals lost $CD4^+$ T cells through the 73 weeks postchallenge observation period (Figs. 5C and 5D).

Persistence of challenge virus and viral DNA in lymph nodes: Specificity and sensitivity of primers. To evaluate the specificity and sensitivity of the primers (Fig. 6), we used DNA from CEM174 cells infected with vaccine-I,

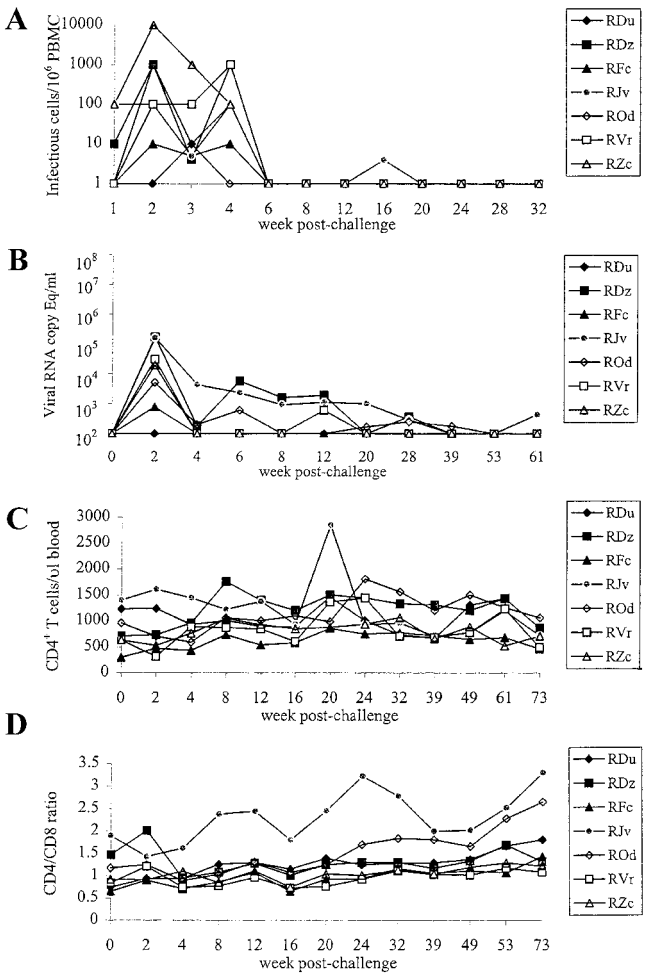

FIG. 5. Cell-associated virus burden, plasma viral RNA loads, $CD4^+$ T cell profile and CD4/CD8 ratio in seven vaccinated macaques challenged with cocktail of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$. Seven macaques, RDu, RDz, RFc, RJv, ROd, RVr, and RZc, were immunized sequentially once with vaccine-I followed by two immunizations with vaccine-II, and challenged intrarectally twice, 1 day apart, with mixture of three pathogenic viruses. Cell-associated viral burdens (A), viral RNA loads in plasma (B), $CD4^+$ T cell counts (C), and CD4/CD8 ratio were determined for a total 73 weeks, postchallenge period, as described in legend of Figure 4.

vaccine-II, $SHIV_{KU-2}$, $SHIV_{89.6}P$, and SIVmacR71/17E, respectively, as templates and performed PCR using primers shown in Table 1. Each set of primers specifically amplified its respective challenge virus and did not amplify either of the two vaccine viruses (Fig. 6). $SHIV_{KU-2}$ and $SIV_{mac}R71/17E$ primers detected one copy of viral genome in 10^5 cells, whereas $SHIV_{89.6}P$ primers detected one copy of viral genome in 10^4 cells (results not shown).

Since challenge viruses and viral RNA had become undetectable in both PBMC and plasma, respectively, of the vaccinated animals, we sought to determine whether DNA of the viruses persisted in lymph nodes collected at weeks 61 and 82 after challenge. Further, using isolate-specific PCR amplification, we sought to assess which viruses were present. Macaque RDu did not have detectable DNA of any of the viruses at either time point. $SHIV_{KU-2}$ DNA was present in the six other macaques at week 61 and was still present in four of the six at week 82. Macaque RFc and RVr became negative for $SHIV_{KU-2}$ DNA during the intervening period. $SHIV_{89.6}P$ DNA was detectable in four (RDz, RJv, ROd, and RZc) of the seven vaccinates at week 61, and by week 82, this DNA remained detectable in lymph nodes of only two animals (RDz and ROd). Thus, five of the seven vaccinates had no detectable DNA of this virus in lymph nodes. $SIV_{mac}R71/17E$-specific DNA was detectable at both time points in only one of the seven macaques (RJv) (Fig. 7). At week 82 we examined CD8-depleted lymph node cells for the presence of infectious virus. Infectious virus was recovered from three of the seven vaccinates. Two of the three macaques yielded two challenge viruses (RJv, $SHIV_{KU-2}$ and $SHIV_{89.6}P$; ROd, $SHIV_{KU-2}$, and $SIV_{mac}R71/17E$). The third macaque (RZc) yielded only $SHIV_{KU-2}$ (results not shown).

Immunological studies

Neutralizing antibody responses during the postchallenge period. At the time of challenge, all seven vaccinated animals had neutralizing antibodies against vaccine-I virus, and six of the seven had neutralizing antibodies against vaccine-II and $SHIV_{KU-2}$. The challenge with pathogenic viruses resulted in a strong boost in antibodies against vaccine-I, vaccine-II, and $SHIV_{KU-2}$ (Fig. 8). These antibodies persisted throughout the 78-week postchallenge observation period. The seventh macaque did not have detectable neutralizing antibodies

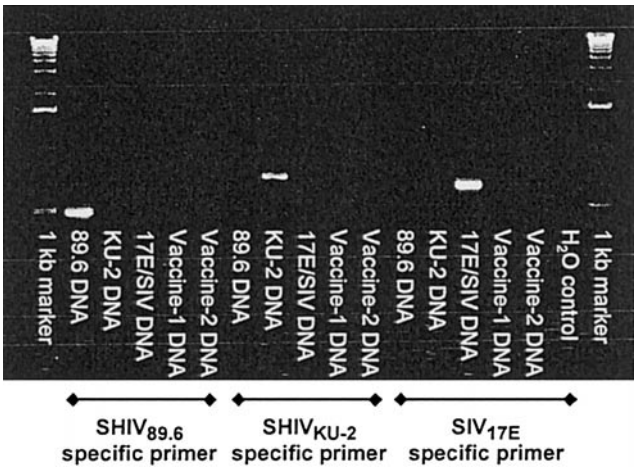

FIG. 6. Specificity of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$ primers. CEM cells were infected with vaccine-I, vaccine-II, $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$. Three days following infection cells were harvested, and total cellular DNA extracted as described under Materials and Methods. Total cellular DNA (0.5 μg) was used as template in a nested/heminested PCR using different sets of primers shown in Table 1. Aliquots of second round PCR electrophoresed on 1.5% agarose gel, and DNA was visualized by ethidium bromide staining. Lanes are marked in terms of DNA template and primers used for the PCR.

TABLE 1

Sequences of the Primers Used for Amplification of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and SIVmacR71/17E

Target virus	PCR	Primer	Sequence	Product size[a] (bp)
$SHIV_{KU-2}$	Ist round	Sense	5′-[6825]GAT-AGA-CTA-ATA-GAA-AGA-GCA-GAA-GAC[6851]-3′	
		Antisense	5′-[7797]TGG-TCC-TCT-CTG-GAT-ACG-GAT-T[7776]-3′	
	II round	Sense	5′-[7098]CCC-ACA-AGA-AGT-AGT-ATT-GGT-[7118]3′	
		Antisense	5′-[7797]TGG-TCC-TCT-CTG-GAT-ACG-GAT-T[7776]-3′	699
$SHIV_{89.6}P$	Ist round	Sense	5′-[6303]GAT-AGA-CTA-ATA-GAA-AGA-GCA-GAA-GAC[6329]-3′	
		Antisense	5′-[7669]GAT-GAA-CAT-CTA-ATT-TGT-CCT-G[7648]-3′	
	II round	Sense	5′-[7168]CTG-AAG-ATT-TCA-CAG-ACA-ATG-T[7189]-3′	
		Antisense	5′-[7669]GAT-GAA-CAT-CTA-ATT-TGT-CCT-G[7648]-3′	501
SIVmacR71/17	Ist round	Sense	5′-[6854]GTA-AGT-ATG-GGA-TGT-CTT-GG[6873]-3′	
		Antisense	5′-[7748]TCC-TAT-TAT-CCC-TAC-CAT-GCC-AG[7726]-3′	
	II round	Sense	5′-[6877]TCA-GCT-GCT-TAT-CGC-CAT-CT[6896]-3′	
		Antisense	5′-[7515]GAC-TCT-TTG-ATA-ACA-GAA-GTG-TTA-CAG[7489]-3′	638

[a] Product size indicates final molecular weight of the product amplified after second round PCR.

against vaccine-II and $SHIV_{KU-2}$ at the time of challenge, but it developed these antibodies to the two viruses after challenge. One of the seven vaccinated animals had $SHIV_{89.6}P$-specific neutralizing antibodies at the time of challenge, and this animal showed an anamnestic response to this virus after challenge. Five others developed these antibodies only after challenge. Interestingly, macaque RDu that showed the strongest resistance to replication of the challenge viruses failed to develop neutralizing antibodies against $SHIV_{89.6}P$. Similarly none of the seven vaccinated animals had anti-$SIV_{mac}R71/17E$-neutralizing antibodies at the time of challenge and they did not develop these antibodies during the 78-week postchallenge observation period (Fig. 8), even though they controlled replication of this virus very efficiently. Neither of the two control animals developed any neutralizing antibodies against any of the three challenge viruses (results not shown).

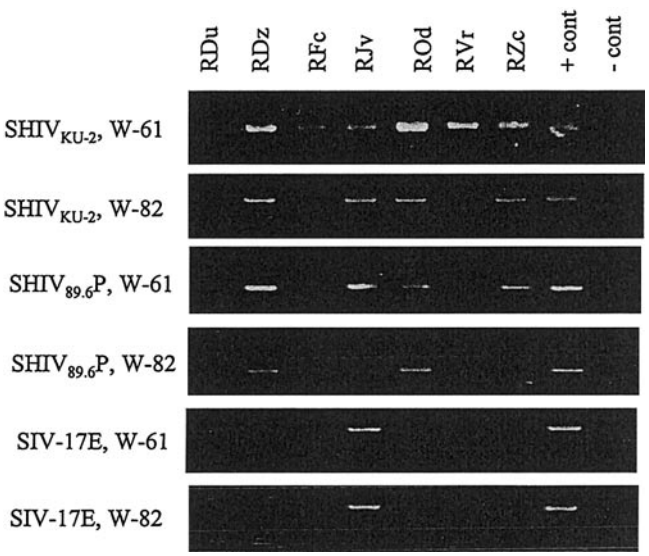

FIG. 7. Persistence of challenge virus-specific DNA in lymph nodes of vaccinated animals challenged with cocktail viruses. Lymph node biopsies were obtained from seven vaccinated macaques 61 and 82 weeks after challenge. Total cellular DNA was extracted, and 0.5 μg DNA was used in each PCR using primers specific for $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$. Five microliters of second round PCR product was electrophoresed on a 1.5% agarose gel and DNA bands were visualized by ethidium bromide staining. Boxes on left marked time and primer specificity, whereas animal names are mentioned on the top.

CTL activity during the postchallenge period. CTL activity against vaccine-II and the three challenge viruses was examined 3, 12, 28, 50, 61, and 70 weeks after challenge. All seven vaccinated animals developed increased CTLs against vaccine-II, $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$, and these responses were directed against Env, Gag, Pol, and Nef proteins (Table 2). The CTLs were present in all seven animals and were already present at week 3 after challenge when the first assay was done. There was no definitive correlation between percentage virus-specific lysis and viral loads in PBMC or plasma. Nonetheless, these cells persisted for at least 70 weeks after challenge. The two control animals did not develop challenge virus-specific CTLs (results not shown).

Quantitation of virus specific-$CD4^+$ and $CD8^+$ T cell responses after challenge. The virus-specific $CD4^+$ and $CD8^+$ T cells were quantified by intracellular IFN-γ staining in response to stimulation with irradiated autologous B-LCL after overnight infection with recombinant vaccinia viruses expressing HIV-1Env, SIV-Gag, SIV-Pol, and SIV-Nef. This assay was performed once during the vaccine phase (60 weeks after the second vaccine-II inoculation) and at weeks 8, 24, 39, and 74 after challenge with the pathogenic viruses. The results of these experiments are shown in Fig. 9. Virus-specific $CD4^+$ and $CD8^+$ T cells were detectable in all seven vaccinated animals even at 60 weeks after the last immunization. The virus-specific $CD4^+$ T cells

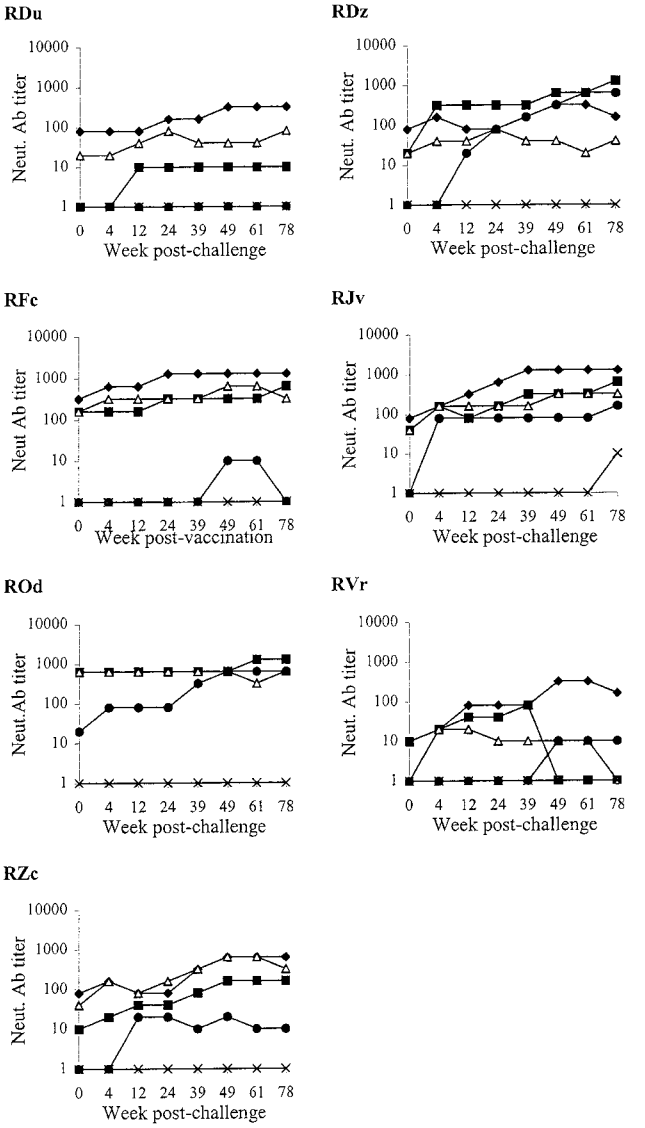

FIG. 8. Persistence of neutralizing antibodies in seven vaccinated macaques challenged with cocktail of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$. Neutralizing antibody titers were determined by preincubating different dilutions of sequentially collected plasma samples in quadruplicate with different vaccine and challenge viruses, followed by culture with C8166 cells for 7 days. The wells were scored for CPE and results are presented as end-point neutralizing antibody titers against vaccine-I (◆), vaccine-II (□), $SHIV_{KU-2}$ (△), $SHIV_{89.6}P$ (●), and $SIV_{mac}R71/17E$ (×).

ranged between 0.8 and 1.34% of the total $CD4^+$ T cell population, and virus-specific $CD8^+$ T cells ranged between 0.8 and 1.56% of the total $CD8^+$ T cells in the PBMC of vaccinated animals. Following challenge, the animals developed massive anamnestic responses. At week 8, postchallenge, approximately 10–30% of the total $CD4^+$ and $CD8^+$ T cells were virus-specific. These virus-specific $CD4^+$ and $CD8^+$ T cell populations declined gradually, and at the time of last quantification, 74 weeks postchallenge, the frequency of these cells ranged between 0.9 and 3.1%.

Vaccinated animals developed long-term soluble virus inhibitory factors. At 3 and 61 weeks after challenge of the animals, supernatant fluids of cultures of PBMC from all seven vaccinated animals were tested for inhibition of replication of vaccine-II, $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$. Results of these experiments are shown in Fig. 10. The supernatant fluids from all seven vaccinated animals completely inhibited replication of all the four viruses. The virus inhibition was complete and these factors were present at least for 61 weeks when the last assay was done. Similar fluids from infected control animals did not have soluble virus inhibitory factor(s).

DISCUSSION

We used two nonpathogenic SHIVs from which accessory genes had been deleted as live-attenuated vaccines, in an effort to protect rhesus macaques against mucosal challenge with a mixture of homologous and heterologous pathogenic SHIV and SIV isolates. Despite the absence of direct virological evidence of productive replication by either of the two vaccine viruses, the sequential immunization protocol resulted in infection and development of long-lasting cellular and humoral immune responses in all of the animals. These responses were detectable as long as 14 months following the last vaccine administration. Vaccine-induced responses curtailed replication of the challenge viruses and helped in reduction of concentrations of their RNA in plasma of all of the animals. Further, levels of challenge virus DNA in lymph nodes of several of the immunized animals were reduced to levels below the assay detection thresholds.

We had shown previously that vaccine-II, but not vaccine-I, conferred long-term protection against AIDS induced by homologous pathogenic virus $SHIV_{KU}$. The protection achieved in the vaccine-II group was associated with transiently productive replication of the vaccine virus. Although vaccine-I-induced immune responses did not confer indefinite protection against pathogenic $SHIV_{KU}$ given as challenge, the induced responses nevertheless blunted the replication of challenge virus and delayed the progression of animals to clinical disease state at least by 1 year (Stipp *et al.,* 2000). On the other hand, animals infected with vaccine-II developed limited productive infection and these animals resisted the disease caused by pathogenic challenge virus and have remained healthy for nearly 4 years postchallenge (Joag *et al.,* 1998b; Silverstein *et al.,* 2000; Kumar *et al.,* unpublished observations). In the present study we sought to address whether prior immunization with vaccine-I would curtail the replication of vaccine-II, whether the protective efficacy of vaccination would be retained as observed earlier (Joag *et al.,* 1998b; Silverstein *et al.,* 2000), and whether the protection would be effective against pathogenic heterologous viruses (Silverstein *et al.,* 2000). In seven animals immunized with the sequen-

TABLE 2

Long-Term CTLs in Macaques Immunize with Vaccine-I Followed by Vaccine-II and Challenged with Mixture of $SHIV_{KU-2}$, $SHIV_{89.6}P$ and SIV

Macaque	Week postchallenge	Percentage specific lysis against[a]							
		Control[b]	$SHIV_{KU-2}$[c]	$SHIV_{89.6P}$[c]	SIV[c]	HIV-1Env[d]	SIV-Gag[d]	SIV-Pol[d]	SIV-Nef[d]
RDu	3	0	52.1	19.2	37.6	34.1	24.6	9.3	ND[e]
	12	0	43.6	30.5	32.5	16.1	16.2	7.7	50
	28	0.7	66.7	67.8	51.2	20.9	47.4	42.1	31.6
	50	0	16.1	41.7	39.9	ND	ND	ND	ND
	61	4	46	48.8	37.5	33.7	39.9	60	30.3
	70	0	44	32.5	36.8	33.7	28.1	68	36.5
RDz	3	5.9	42.7	26.3	39.7	38.1	28.3	24.5	ND
	12	0	53.3	26.1	38.3	38.9	38.9	40.1	34.7
	28	0.2	48.8	36.1	80.2	34.3	44.1	28.3	30
	50	0	30.2	29.7	27.9	ND	ND	ND	ND
	61	3.1	55.6	43.7	51	42.7	36.5	30.5	42.7
	70	0.2	37.5	54.2	40.9	50.8	27.3	30.7	61.5
RFc	3	2.6	35	34.7	62.6	15.4	15.8	12	ND
	12	0	36.9	28.5	31.3	26.8	33.5	23.7	24.2
	28	1.7	70.2	53.1	45	ND	35.8	68.8	14.1
	50	0	14.5	11.5	15.2	ND	ND	ND	ND
	61	0	55.7	58.1	50.1	75.4	63.5	44.6	57
	70	0	48.4	52.6	44.4	37.5	43.4	44.5	25
RJv	3	2.4	32.8	50.6	54.2	26.6	16.1	29.7	ND
	12	0	34.8	33.3	22.2	12	25	27.9	31.9
	28	0	33.2	44	57.9	34.9	31.5	34.2	42.5
	50	3.5	45.6	46.2	29.8	ND	ND	ND	ND
	61	0.2	51.8	50.9	58.5	60.8	80.3	33.9	63.3
	70	0	48.4	27.7	20	32.4	51.7	40.6	38.7
ROd	3	1.4	44.2	28.2	43.4	17.1	2.3	25.3	ND
	12		48.2	17.3	44.8	15.6	35.4	21.2	31
	28	0	35.7	32	61	50.5	46.5	47.7	40.5
	50	0	32.7	43.3	19.7	ND	ND	ND	ND
	61	0	52.8	67.1	55.6	33.1	58.6	67.4	41.9
	70	2.3	26.3	50.5	38.4	45.3	39.6	40.6	50.1
RVr	3	0	66.6	28.8	26.2	50.4	29.7	50.8	ND
	12		45.7	38.4	47.1	44.7	32.5	13.8	23.1
	28	1	40.2	37.6	39.2	32.3	68.7	43.3	36.8
	50	0	54.4	ND	15	ND	ND	ND	ND
	61	6.6	59.1	50.1	70.2	34.7	33.7	57.8	38.7
	70	0.5	36.6	45.6	18.1	41.6	44.9	38.3	23.3
RZc	3	0	40.8	21.4	59.6	26.1	12	20.3	ND
	12	0	34.9	37.1	55	27.7	33.9	37.8	17.8
	28	0	51.8	47	54.5	39.9	55.8	37.3	55
	50	ND	ND	ND	ND	ND	ND	ND	ND
	61	2.8	63.5	55.2	47.3	49.5	61.7	35.1	48.4
	70	1.1	53.5	42	45.2	31.4	33.3	39.1	32.3

[a] PBMC effectors from different animals were tested against different targets at effector/target ratio of 80/1, 40/1, 20/1, and 10/1. The results shown here are specific lysis obtained at effector/target ratio of 40/1. The mean spontaneous counts in these experiments ranged between 50 and 860, and mean maximal count ranged between 330 and 3650.

[b] Specific lysis against control targets represents either uninfected autologous $CD4^+$ T cell clone or autologous B-LCLs mock-infected and used as targets in 4-h chromium release assay.

[c] Autologous $CD4^+$ T cell clones were infected with $SHIV_{KU-2}$, $SHIV_{89.6}P$, or SIV for 3 days, labeled with [^{51}Cr], and used in cytotoxicity assays.

[d] Autologous B-LCLs were infected overnight with recombinant vaccinia virus expressing HIV_{Env} or SIV_{Gag} or SIV_{Pol} or SIV_{Nef} and used as targets.

[e] Not done.

tial vaccine regimen, there was no evidence of infectious virus in the PBMC of any. However, the animals developed demonstrable vaccine-II-specific $CD4^+$ and $CD8^+$ T cells, and functional CTL responses against vaccine-II were detectable by intracellular IFN-γ staining and chromium release assays, respectively. Although no infectious vaccine virus was recovered from the PBMC of the vaccinated animals, the presence of CTL responses in these animals suggested the persistence of low-level viral replication. Vaccine virus-specific CTLs caused spe-

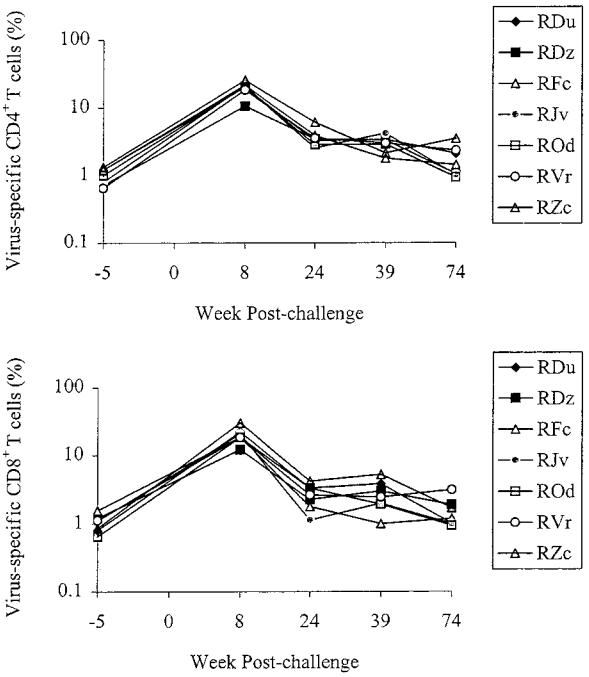

FIG. 9. Quantitation of virus-specific $CD4^+$ and $CD8^+$ T cells in macaques immunized with vaccine-I and vaccine-II and challenged with cocktail of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$. Two million PBMC, collected at 5 weeks before challenge and 8, 24, 39 and 74 weeks after challenge, were stimulated with UV-irradiated autologous B-LCL infected with mixture of recombinant vaccinia viruses expressing HIV-1Env, SIV-Gag, SIV-Pol, and SIV-Nef for 12–14 h with 5 μg/ml brefeldin-A for the last 6 h. The cells were then stained with antibodies against CD3, CD4, CD8, and IFN-γ, and analyzed on FACS calibur. Percentage of $CD4^+$ IFN-γ^+, and $CD8^+$ IFN-γ^+ T cells with mock-stimulation (<0.5%) was subtracted from percentage of viral proteins-specific $CD4^+$, IFN-γ^+, and $CD8^+$ IFN-γ^+ T cells to calculate percentage population of specific cells.

cific lysis of autologous $CD4^+$ T cells infected with $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$, underscoring the fact that CTL activity was either directed against conserved epitope(s) in the envelope protein or the other proteins common to the virus strains, Gag, Pol, and Nef.

All vaccinated animals developed vaccine-I and vaccine-II Env-specific binding antibodies that cross-reacted with the envelope protein of homologous pathogenic virus $SHIV_{KU-2}$, and to a lesser extent cross-reacted with $SHIV_{89.6}P$ envelope. However, vaccine-specific antibodies did not cross-react with the envelope of $SIV_{mac}R71/17E$, presumably because of the extensive sequence disparity between the envelope protein of the SIV and other viruses. Likewise, six of the seven challenged vaccinates developed neutralizing antibodies against both of the vaccine viruses. Five vaccinates developed neutralizing antibodies against $SHIV_{KU-2}$, and two developed neutralizing antibodies against $SHIV_{89.6}P$, respectively, suggesting that some of the neutralization epitope(s) were conserved between vaccine-I and -II, and $SHIV_{KU-2}$ and $SHIV_{89.6}P$. None of the animals developed neutralizing antibodies against SIV. This lack of neutralization response again likely reflected the heterogeneity between the envelopes of SHIVs and SIV.

The importance of virus-specific $CD8^+$ T cell responses has been indirectly shown in several studies in HIV-infected individuals with the demonstration that virus-specific CTLs correlated with decline of plasma virus burdens (Day *et al.,* 2001; Walker and Korber, 2001; Goulder *et al.,* 2001; Altfeld *et al.,* 2001; Walker and Rosenberg, 2000). Similar correlations have been observed in macaques infected with SIV and SHIV (Johnson and Desrosiers, 1998; Cromwell *et al.,* 2000; Kaur *et al.,* 2000; Joag *et al.,* 1998b; Silverstein *et al.,* 2000; Kumar *et al.,* 2001a,b), in which CTLs (Kaur *et al.,* 2000; Joag *et al.,* 1998b; Silverstein *et al.,* 2000; Kumar *et al.,* 2001a,b) correlated inversely with decline in virus replication. A more direct role for the CTLs was shown in studies in which depletion of $CD8^+$ T cells in SIV/SHIV-infected macaques resulted in dramatic increases in plasma viremia (Metzner *et al.,* 2000; Schmitz *et al.,* 1999; Jin *et al.,* 1999). The present study provides further insights into the importance of T cell mediated responses. The vaccine viruses induced both humoral and cellular immune responses that were present throughout the prechallenge period, but the CMI responses probably were of greater importance in controlling the challenge viruses given subsequently. All animals developed vaccine virus-specific neutralizing antibodies that were detectable at the time of challenge, but these antibodies did not cross-neutralize one or more of the challenge virus(es). In contrast, vaccine-induced CTLs and soluble inhibitory factor(s) showed activity against not only vaccine viruses but also against all the challenge viruses, suggesting

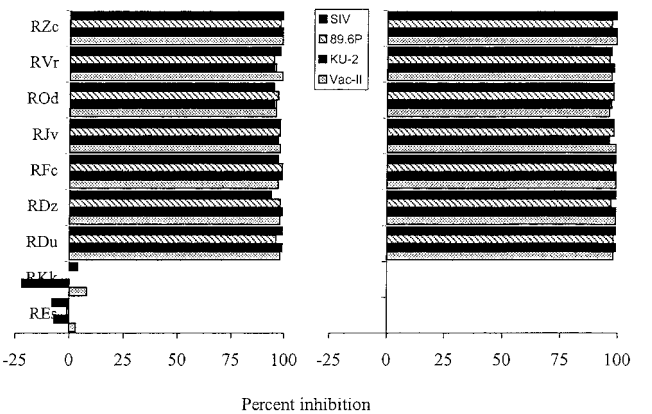

FIG. 10. Postchallenge antigen-induced soluble viral inhibitory factor(s) from PBMC of seven vaccinated and two control macaques. The soluble factors were prepared by cocultivation of PBMC, obtained at 3 (A) and 61 (B) weeks after challenge, with UV-irradiated autologous HS-$CD4^+$ T cell clones infected with mixture of vaccine and challenge viruses, as described under Materials and Methods. Culture supernatants were collected on day 7, filtered through 0.22-μM membrane, and tested for their ability to block *in vitro* replication of vaccine-II, $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$ in CEM cells. Virus replication in CEM cells was determined by measuring RT activity in 30 μl supernatants. The results presented here depict inhibition in percentage considering virus production in the absence of PBMC supernatants as 100%.

that the major role for these cellular immune responses was containment of challenge virus replication.

The quantitative dissection of virus-specific cellular immune responses has been revolutionized by several recently introduced technologies. These include development of tetrameric complexes of major histocompatibility complex class I (MHC-I) or MHC-II glycoproteins and viral peptides for the direct-flow cytometric staining of responding lymphocyte populations (Kuroda *et al.,* 1998; Barouch *et al.,* 2001; Callan *et al.,* 1998; Gallimore *et al.,* 1998), ELISPOT (Goulder *et al.,* 2001; Lalvani *et al.,* 2001; van Baarle *et al.,* 2001; Scheibenbogen *et al.,* 2000; Shacklett *et al.,* 2000; Larsson *et al.,* 1999; Corne *et al.,* 1999; Dunbar *et al.,* 1998; Mayer *et al.,* 1996), and intracellular cytokine staining (Goulder *et al.,* 2001; Marshall *et al.,* 2001; Belz *et al.,* 1998; Stevenson *et al.,* 1998). The tetramer technology can only be used within the context of MHC-I and specific viral epitopes, whereas ELISPOT and intracellular cytokine (IFN-γ) staining can be used for studies in outbred animals, without any prior knowledge of MHC restriction elements, or the sequences of defined CTL or Th epitopes. Furthermore, several studies have shown comparable profiles between intracellular IFN-γ staining and tetramer staining (Flynn *et al.,* 1998; Murali-Krishna *et al.,* 1998). In view of the limited knowledge regarding macaque MHC and T-helper/CTL epitopes, ELISPOT and intracellular IFN-γ staining offer a better means to quantitate virus-specific CTL or T-helper responses in outbred macaques. We used intracellular IFN-γ staining to quantify virus-specific cellular responses. Our study indicated massive anamnestic $CD4^+$ and $CD8^+$ T cell responses as measured by staining of PBMC for their ability to secrete interferon-γ in response to stimulation with viral antigen. The presence of 10–30% virus-specific $CD4^+$ and $CD8^+$ T cells pointed to a clonal expansion of virus-specific T cells after challenge. The massive anamnestic response has also been reported in another study (Allen *et al.,* 2000b).

Earlier findings by several groups (Walker *et al.,* 1986) showing that soluble factors secreted by $CD8^+$ T cells (CD8-SF) can block replication of the virus suggested this as a possible mechanism contributing to control of virus replication in our study. This noncytolytic function of $CD8^+$ T cells has also been shown in HIV-1-infected individuals and SIV-infected animals (Aubertin *et al.,* 2000; Lehner *et al.,* 1996, 1999; Wang *et al.,* 1998; Walker *et al.,* 1986; Garzino-Demo *et al.,* 1999). Whereas earlier reports had used nonspecific stimulation to identify CD8-SF, we adopted the more clinically relevant strategy to ask whether viral antigen could be important for inducing production of inhibitory soluble factors. In a recent report antigen-induced chemokine production in HIV-positive individuals has been inversely correlated with the progression of clinical disease (Garzino-Demo *et al.,* 1999). Here, we have shown that vaccine viruses induced inhibitory factors that blocked replication of not only the vaccine virus but also the challenge viruses. The ability of PBMC to secrete these factors was sustained for a long time and might have contributed to the elimination of challenge viruses, after the latter had established infection in lymph nodes.

Another interesting finding that emerged from this study was that although $SIV_{mac}R71/17E$ is the most divergent from the vaccine viruses in its nucleotide sequences, it was the easiest virus to be controlled. At the time of challenge, vaccinated animals had neither SIV-Env-specific CTL nor anti-SIV neutralizing antibodies. Only SIV core-specific CTL and SIV-specific soluble inhibitory factors were detectable at the time of challenge. On the other hand, $SHIV_{KU-2}$ was the challenge virus that had closest sequence homology to the vaccine viruses. At the time of challenge, all seven animals not only had CTLs directed against Env and core proteins and $SHIV_{KU-2}$-specific inhibitory factors, but several of them also had $SHIV_{KU-2}$-specific neutralizing antibodies. In spite of this, $SHIV_{KU-2}$-specific DNA persisted in lymph nodes of four of the seven animals at 81 weeks after challenge, and two of the seven animals are still harboring replication-competent $SHIV_{KU-2}$ at this time point. The reason for this is unclear but may relate to intrinsic properties of $SHIV_{KU-2}$ that made it harder to be controlled/eliminated compared to $SHIV_{89.6}P$ and $SIV_{mac}R71/17E$.

In summary we provide evidence that long-term protection against simultaneous challenge with homologous and heterologous SHIV and SIV is possible. The protection seen in these animals can be characterized not only by prevention of disease and reduction of viral loads in plasma but by progressive reduction over time of challenge virus DNA in PBMC and/or lymph nodes, in some instances to below detectable levels. This effect was probably mediated by a combination of neutralizing antibodies, CTLs against different challenge viruses, and soluble inhibitory factors. Similar results of DNA vaccine-induced reduction of challenge virus replication have been reported by others (Amara *et al.,* 2001; Barouch *et al.,* 2000) but whether these vaccine strategies also resulted in progressive elimination of challenge virus DNA as seen in this study is not known as yet.

The final point emerging from this study was that a graded course of immunization first with a more attenuated virus followed by a less attenuated one may be an effective way of reducing replication of highly effective live vaccine viruses and thus reducing the chances of reversion of vaccine viruses into pathogenic strains. This approach, of using prior immunization to enhance the safety of an attenuated virus vaccine strain, is broadly similar to the current recommendation for pediatric polio immunization, in which children receive killed polio virus vaccine prior to being vaccinated with live-attenuated polio virus vaccine (Wadsworth, 1999). Live-attenuated vaccines have been among the more efficacious vaccines developed, and in nonhuman primate studies, live-

attenuated SIV vaccines (Desrosiers, 1995; Johnson and Desrosiers, 1998; Johnson *et al.,* 1999), and SHIV vaccines (Joag *et al.,* 1998b; Silverstein *et al.,* 2000), and this study has shown levels of efficacy that have not been matched by other vaccine approaches. However, safety is always a major concern for live-attenuated vaccines and this concern is amplified in contemplating a live-attenuated vaccine for a pathogen such as HIV, for which an uncontrolled infection could be fatal (Baba *et al.,* 1999). The variation on the sequential immunization approach described here, or the fact that the live vaccine virus failed to become pathogenic after serial passage in animals (Mackay *et al.,* 2002), reduced but did not eliminate concerns about use of live virus vaccines against HIV. Nevertheless, these studies provide further proof of concept data of the potential to which nonlive vaccines could be aimed.

MATERIALS AND METHODS

Viruses

The construction of vaccine-I, vaccine-II, and derivation of $SHIV_{KU-2}$ has been previously described (Joag *et al.,* 1998a,b). Vaccine-I was derived from nonpathogenic SHIV-4, by deleting 60 bp from *vpu* and 205 bp from *nef.* Vaccine-II was derived from another nonpathogenic virus $SHIV_{PPC}$ by deleting 60 bp from *vpu* (Joag *et al.,* 1998a). $SHIV_{KU-2}$ was derived from nonpathogenic SHIV-4 first by serial passage in young pigtailed macaques (Joag *et al.,* 1996) followed by passage in rhesus macaques (Joag *et al.,* 1998b). Stock preparations of these viruses were propagated in macaque PBMC. Infectivity of stocks of the three viruses was assayed in C8166 cell cultures and had titers of 10^4 (vaccine-I), 1.6×10^4 (vaccine-II), and 6.3×10^4 $TCID_{50}$/ml ($SHIV_{KU-2}$). $SHIV_{89.6}P$ was kindly provided by Dr. N. Letvin (Reimann *et al.,* 1996a,b), and stock virus was prepared by cultivation in mitogen-stimulated macaque PBMC. The titer in C8166 cells was 4×10^8 TCID/ml. $SIV_{mac}R71/17E$ is a neurovirulent variant of $SIV_{mac}251$ (Sharma *et al.,* 1992). *Herpesvirus papio* stock was obtained by collecting supernatant fluid from a persistently infected cell line S594 (provided by Dr. N. Letvin) (Rabin *et al.,* 1977). *H. saimiri* stock was obtained from ATCC and propagated in owl monkey kidney cell cultures as described previously (Kumar *et al.,* 1999). The recombinant vaccinia viruses that were used to infect target cells included vAbT299 (expressing $HIV\text{-}1_{BH\text{-}10}$ENV lacking 19 amino acids starting from position 42–60 at the amino-terminus), vT60 ($SIV_{mac}239$ Env Δ leader), vAbT252 ($SIV_{mac}251$-GAG), vAbT258R ($SIV_{mac}251$-POL), vAbT306 ($SIV_{mac}239$ Nef), and NYCBH (wild-type vaccinia virus). These viruses were kindly provided by Drs. D. Panicali and G. Mazzara, Therion Biologics, Cambridge, MA. The recombinant vaccinia viruses were propagated in HeLa cell cultures. All virus stocks were maintained at −80°C except for the challenge virus stocks which were stored in liquid nitrogen.

Vaccination of macaques and challenge with $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$

Nine 3- to 5-year-old rhesus macaques (*Macaca mulatta*) were obtained from the Yerkes Primate Center, Atlanta, GA and maintained in the AAALAC-approved Animal Facility of the University of Kansas Medical Center. Seven animals were inoculated subcutaneously, close to the inguinal and axillary lymph nodes, with 1 ml of vaccine-I. Four months later, the animals received two doses of 1 ml of vaccine-II (10^4 $TCID_{50}$), given orally 1 day apart. Three months following this, the animals were administered a further inoculation of vaccine-II (10^4 $TCID_{50}$), subcutaneously, because of failure to detect infectious virus in PBMC following the oral inoculations. Fifty-eight weeks after the third immunization, the vaccinated and two unvaccinated control animals were challenged twice, 1 day apart, by intrarectal route with a mixture of undiluted $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$ stocks. The animals were bled regularly to monitor $CD4^+$ T cell profiles, cell-associated as well as cell-free viral loads in the blood, and cellular and humoral immune responses to the different viruses.

Flow cytometry

Lymphocyte subset profiles were determined by staining for CD3, CD4, and CD8 surface markers, using the whole-blood lysis technique (Wyand *et al.,* 1996). Briefly, 10 μl of the antibody mix against CD3, CD4, and CD8 (Becton–Dickinson) was added to 100 μl of whole blood and incubated for 1 h in the dark. Lysing solution (Becton–Dickinson) was then added and the samples were incubated for another 10 min at room temperature. Stained cells were fixed with 0.5% paraformaldehyde and analyzed in a flow cytometer (Becton–Dickinson FACS Calibur).

Viral load measurements

Plasma viral RNA levels were determined by real-time reverse transcriptase PCR (ABI Prism 7700-sequence detection system, Perkin–Elmer) essentially as described previously (Lifson *et al.,* 2001). The sensitivity of this assay is 100 copy Eq/ml of plasma. The frequency of infectious cells in blood was determined by inoculation of serial 10-fold dilutions of PBMC into cultures of C8166 cells that were then observed for the development of cytopathic effects (CPE) (Joag *et al.,* 1998a,b). Results of this infectious cell assay (ICA) are expressed as the number of infectious cells per 10^6 PBMC.

Recovery of infectious virus from PBMCs and lymph node cells

Two million LNMC $CD4^+$ T lymphocytes, enriched by negative selection, were stimulated with PHA (1 μg/ml) for 2 days in 2 ml R-10 (RPMI-1640 containing 10% FBS,

5 mM $NaHCO_3$, and 50 μg/ml gentomycin). The cells were washed and cultured in R-10/IL-2 (R-10 containing 50 IU/ml recombinant interleukin-2) for 5 days. The supernatant fluids were tested for viral cytopathocity in C8166 cultures.

Detection of challenge virus DNA in lymph nodes of challenged animals

DNA extracted from lymph nodes from challenged animals was screened for the presence of DNA of different challenge viruses by PCR, using primers specific for the envelope sequences of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$, respectively. The sequence of the primers for first- and second-round amplifications, the location of the primers in the viral genome, and the expected size of the PCR products are shown in Table 1. Genomic DNA (0.5 μg) was used in the PCR mixture containing 4 mM $MgCl_2$, 200 μM each of the four deoxynucleoside triphosphates, 100 pM each oligonucleotide primers, and 2.5 U of *Taq* polymerase (Perkin–Elmer Cetus, Norwolk, CN) in a hot start PCR. The template was denatured at 95°C for 3 min, and PCR amplification performed with an automated DNA thermal cycler (Perkin–Elmer Cetus) for 35 cycles of denaturation at 92°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 3 min. The reaction was completed after 10 min of primer extension at 72°C. Two microliters of resultant product was used in the second-round PCR, using primers described in Table 1 and conditions as described above for the first-round PCR. Following the second round of amplification, a 5-μl aliquot was electrophoresed on a 1.5% agarose gel, and the bands visualized by staining with ethidium bromide.

Measurement of envelope-specific antibody

Plasma samples from vaccinated monkeys were analyzed for their reactivity to native viral glycoprotein in a concanavalin A (Con A) ELISA, originally developed by Robinson and co-workers (Robinson *et al.,* 1990) and later adapted to SIV and SHIV system by Cole and co-workers (Cole *et al.,* 1997a,b, 2000). Triton X-100 treated and density gradient-purified virus (vaccine-I, vaccine-II, or $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$) was used as viral antigen and plasma from different animals were tested for the binding antibody titers as described previously (Kumar *et al.,* 2000).

Determination of neutralizing antibodies against vaccine viruses

Neutralizing antibody titers in the plasma were determined by adding serial twofold dilutions of heat-inactivated plasma, prepared in R-10 to flat-bottom 96-well plates, using four wells per dilution. Eight $TCID_{50}$ of vaccine-I, vaccine-II, or $SHIV_{KU-2}$ viruses were then added to each well and the plates were incubated at 37°C for 1 h, followed by addition of 10^4 C8166 cells to each well. After 1 week of incubation at 37°C, each well was scored for CPE (Kumar *et al.,* 2000, 2001a,b).

Development of lymphoblastoid B cell line (B-LCL)

The B lymphoblastoid cell lines (B-LCLs) were derived by inoculating PBMCs with *H. papio* and maintaining the cells for 2 weeks in the presence of 500 ng/ml of cyclosporin-A (Sigma, St. Louis, MO) in 200 μl R-10. Proliferating cells were transferred to tissue culture flasks and maintained in R-10 throughout the study (Kumar *et al.,* 2000).

Development of autologous $CD4^+$ T cell clones

$CD4^+$ T cells from all animals were immortalized by infection with *H. saimiri.* Briefly, CD8-depleted PBMCs were inoculated with *H. saimiri* and maintained in R-10/IL-2 for 1 week. The cells were then cloned by the limiting dilution method, using irradiated human PBMCs as feeders. Proliferating clones were cultured in R-10/IL2 and characterized by the expression of the CD4 cell-surface marker (Kumar *et al.,* 1999).

Generation of effector T cell populations and CTL assays

Effectors for the CTL assays were obtained by coculturing PBMC from vaccinated and control macaques with UV-irradiated, autologous *H. saimiri* immortalized $CD4^+$ T cells (HS-$CD4^+$ T cells) infected with vaccine-II virus, $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$, respectively (Stipp *et al.,* 2000). After 3 days of coculture, the medium was supplemented with 100 IU/ml of recombinant human interleukin-2 (rIL-2). The cells were restimulated on day 7 with stimulators in a fashion similar as described above. CTL activity was determined in a 4-h chromium release assay using effectors from different animals and autologous targets. The targets included autologous HS-$CD4^+$ T cells infected with one of the vaccine or challenge viruses, and B-LCLs infected with recombinant vaccinia viruses expressing HIV-1-Env, SIV-Gag, or SIV-Pol. Target cells, labeled with 100 μCi ^{51}Cr (specific activity, 962 MBq/ml of sodium chromate, Amersham, Cleveland, OH), were dispensed into 96-well V-bottom plates and effector cells were tested in triplicate at effector/target (E/T) ratios of 80/1, 40/1, 20/1, and 10/1. Chromium release was determined after 4 h of incubation at 37°C by counting 100 μl supernatant from each well in a Gamma counter (Packard, Meriden, CT). Each experiment had three wells containing 2500 targets in medium that provided data for spontaneous lysis while three wells containing targets and 100 μl of 1% SDS provided data for maximal lysis. The percentage specific lysis was calculated as (mean CPM in test wells − mean CPM in spontaneous wells)/(mean CPM in maximal wells − mean CPM in spontaneous wells) × 100. The limitation of having only two

animals in the control group in this study precluded any statistical comparisons between the vaccinated and control animals.

Determination of virus inhibitory factor(s)

Virus-inhibitory factors were determined using a previously described protocol (Garzino-Demo *et al.,* 1999), with some modifications. PBMC from vaccinated and control animals were stimulated with UV-irradiated autologous $CD4^+$ T cells infected with vaccine-II and three challenge viruses. The cells were cultured at a density of $3–3.5 \times 10^6$ per milliliter R-10 medium. Three days after the initial stimulation, rIL-2 was added at a concentration of 50 IU/ml of medium. Supernatant fluids of the cultures from different animals were collected on day 7, centrifuged, and filtered through 0.45-μM membrane.

To assay the effect of inhibitory factor(s), CEM174 cells were inoculated with 10^4 $TCID_{50}$ of vaccine-II, $SHIV_{KU-2}$, $SHIV_{89.6}P$, or $SIV_{mac}R71/17E$, respectively, for 2 h. The unbound virus was removed by washing cells thrice with R-10 medium. The cells were counted, resuspended in R-10 medium, and cultured in triplicate in flat-bottom 96-well tissue culture plates in R-10 medium, with or without the test supernatant fluids from individual animals. Control wells received 200 μl R-10 medium, whereas experimental wells received 100 μl medium and 100 μl supernatant from each animal. On day 7, 30 μl of supernatant fluid from each well was analyzed for virus production by the reverse transcriptase assay as described previously (Kumar *et al.,* 1999).

Quantitation of virus-specific $CD4^+$ and $CD8^+$ T cells by intracellular cytokine staining

Virus-specific $CD4^+$ and $CD8^+$ T cells were quantified by modification of a procedure described earlier (Gauduin *et al.,* 1999). Briefly, 2×10^6 freshly isolated PBMC were stimulated with UV-irradiated autologous B-LCLs that had been inoculated with a mixture of recombinant vaccinia viruses expressing HIV-1Env, SIV-Gag, SIV-Pol, and SIV-Nef for 12–14 h, with addition of 5 μg/ml of brefeldin-A for the last 6 h. The cells were then stained with antibodies against CD3, CD4, CD8, and IFN-γ, and analyzed on FACS calibur. In each case, the percentage of $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ T cells without stimulation or mock-stimulation (0.5%) was subtracted from percentage of viral protein-specific $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ T cells to calculate specific cells.

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